PA 997543

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

April 21, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/393,994***
FILING DATE: ***July 03, 2002***

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WOODSON
Certifying Officer

Please type a plus sign (+) inside this box [+]

Docket No. 6819

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
Marco E.	Bianchi	Viale Abruzzi 4/C 20068 Peschiera Borromeo ITALY

____ Additional inventors are being named on the ___ separately numbered sheet attached hereto.

TITLE OF THE INVENTION (280 characters max)

USE OF HMGB1 PROTEIN AS STEM CELL CHEMOATTRACTANT AND PROLIFERATION PROMOTER IN THE TREATMENT OF THE FIRST STEP OF INFLAMMATION AND OF TISSUE REPAIR

CORRESPONDENCE ADDRESS

Direct all correspondence to:

____ Customer Number

Type Customer Number here

Place Customer Number Bar Code Label here

OR

X Firm *or* Individual Name	Arlene J. Powers Samuels, Gauthier & Stevens, LLP				
Address	225 Franklin Street, Suite 3300				
City, State & ZIP	Boston, Massachusetts 02110				
Country	U.S.	Tel.	(617) 426-9180	Fax	(617) 426-2275

ENCLOSED APPLICATION PARTS ***(check all that apply)***

X Specification *Number of Pages* 18   X Declaration and Power of Attorney

X Drawing(s) *Number of Sheets* 6   X Other (specify) Assignment

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT ***(check one)***

X A check or money order is enclosed to cover the filing fees.

X Applicant Claims Small Entity Status

____ No fee is to be paid at this time.

____ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Order Account Number: 19-0079

FILING FEE AMOUNT <u>$80.00</u>

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

X No.

____ Yes, the name of the U.S. Government agency and the Government contract number are: ____________

CERTIFICATION UNDER 37 C.F.R 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on July 3, 2002 in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10, Mailing Label Number EV011191990US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

[signature]
Signature

Sarah Kennedy
Type or print name of person certifying

Respectfully submitted,

Signature _____ Date: 7/3/02

Arlene J. Powers
Registration No.: 35,985
Telephone: (617) 426-9180
Extension: 110

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

+

Use of the HMGB1 protein as stem cell chemoattractant and proliferation promoter in the treatment of the first step of inflammation and of tissue repair.

HMGB1 (High mobility group 1 protein) is both a nuclear factor and a secreted protein. In the cell nucleus it acts as an architectural chromatin-binding factor that bends DNA and promotes protein assembly on specific DNA targets[1,2]. Outside the cell, it binds with high affinity to RAGE[3] (receptor for advanced glycation endproducts), and is a potent mediator of inflammation[4-6]. HMGB1 is actively secreted by activated monocytes and macrophages[4], and is passively released by necrotic or damaged cells[7-9]. We report here that *Hmgb1*$^{-/-}$ necrotic cells have a greatly reduced ability to promote inflammation, proving that the release of HMGB1 can signal the demise of a cell to its neighbors. Moreover, apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis, and thus fail to promote inflammation even if not promptly cleared by phagocytic cells. In apoptotic cells HMGB1 is firmly bound to chromatin because of generalized histone underacetylation, and is released in the extracellular medium (promoting inflammation) if chromatin deacetylation is prevented. Thus, cells undergoing apoptosis are programmed to withhold the signal broadcast by cells damaged or killed by trauma.

Therefore, though HMGB1 is an abundant component of chromatin, it also has a role as a soluble, extracellular protein. Wang et al. (Science, 285, 248-251, 1999) identified HMGB1 as a late mediator of endotoxin lethality in mice. They showed that monocytes/macrophages stimulated by LPS, TNF or IL-1 secreted HMGB1 as a delayed response. In mice, administration of anti-HMGB1 antibodies attenuated LPS-induced endotoxemia; conversely, injection of HMGB1 caused toxic shock. Moreover, septic patients showed increased serum levels of HMGB1, which correlated with the severity of the infection (US 6,303,321; WO 00/47104).

Subsequently, HMGB1 was also shown to cause acute lung inflammation when administered intratracheally (Abraham et al., J. Immunol., 165, 2950-2954, 2000). Antibodies against HMGB1 decreased lung edema and neutrophile migration, whereas they did not reduce the levels of the other proinflammatory cytokines TNF-α, IL-1α or

macrophage-inflammatory-protein-2 (MIP2). Pituicytes, which provide an important link between the immune and the neuroendocrine s-ystems, release HMGB1 in response to specific stimuli like TNF-α and IL-1, suggesting that HMGB1 also participates in the regulation of neuroendocrine and immune responses to inflammatory processes (Wang et al., Surgery, 126, 389-392, 1999). However, most cells (including lymphocytes, adrenal cells or kidney cells) are not able to secrete HMGB1.

The phenomena investigated by the groups of Wang and Andersson depend from the active (if unconventional) secretion of HMGB1 by specific cells, that respond to specific stimulation by proinflammatory cytokines. HMGB1 secretion requires at least 16 hours after cell stimulation, and thus is a late event in inflammation. The role of secreted HMGB1 is thus to reinforce and prolong inflammation that was initiated by some other event.

In contrast, it has been now demonstrated that HMGB1 released by necrotic cells can be the initial trigger for inflammatory responses, and that released HMGB1 itself can activate inflammatory cells. HMGB1 release and diffusion can take place in a matter of seconds or minutes, and is thus a very early event in inflammation. The ability of certain cell types to secrete HMGB1 without dying must therefore be a type of molecular mimicry: these cells have evolved the ability to secrete the same molecule whose extracellular presence signals tissue damage.

The following succession of events could be envisaged:

Necrosis -> primary HMGB1 release -> inflammatory cell activation -> secretion of cytokines -> further activation and recruitment of inflammatory cells -> secretion of HMGB1 by inflammatory cells -> loop continues with secretion of cytokines and further activation and recruitment of inflammatory cells.

This loop will create a positive feedback, with strong inflammatory responses. The loop must be broken at some point to interrupt inflammatory responses.

Figure legends

Figure 1 Chromatin association of HMGB1 in living and dead HeLa cells. The medium bathing the cells (S) was analyzed by SDS-PAGE alongside with the cells (P). Histones

were visualized by Coomassie staining, HMGB1 by immunoblotting or immunostaining with anti-HMGB1 antibodies, DNA with DAPI. Scale bars, 7.5 μm. a, Living cells expressing HMGB1-GFP, imaged by differential interference contrast and in green fluorescence. b, Interphase cells, after permeabilization. c, Necrotic cells, no permeabilisation. The amount of HMGB1 in the medium was proportional to the number of necrotic cells (about 50%). d, Apoptotic cells, with permeabilization. e, Kinetics of HMGB1 and lactate dehydrogenase (LDH) release from cells undergoing apoptosis and secondary necrosis.

Figure 2 HMGB1 dynamics in living and apoptotic cells. Scale bars, 2.3 μm (a, b) and 3.7 μm (e, f). FLIP imaging (a) and quantitation (c) of HMGB1-GFP in an interphase cell. The area indicated by a circle was repeatedly bleached, and cells were imaged between bleach pulses. A neighbouring cell nucleus was not affected. FLIP imaging (b) and quantitation (d) on a mitotic cell. Bleaching was executed in the cytoplasm (circle), and quantification was done both on a different spot in the cytoplasm, or on a spot on the condensed chromosomes. FLIP imaging in an apoptotic cell (e), and in a cell undergoing apoptosis in the presence of 200 ng/ml TSA (f), and their quantitation (g). h, FLIP quantitation of GFP-HMGN2 in living and apoptotic cells.

Figure 3 Chromatin changes occurring in apoptosis create binding substrates for HMGB1. Scale bar, 9.5 μm. a,b Bacterially made HMGB1 (either labelled with Cy5, a, or unlabelled, b) binds to the chromatin of apoptotic *Hmgb1*$^{-/-}$ fibroblasts, but not to that of non-apoptotic fibroblasts, as visualized by microscopy (a) or Western blotting (b). Histones were visualized by Coomassie staining. c, DNA fragmentation is not responsible for HMGB1 binding to apoptotic nuclei. HeLa cells (expressing a tagged form of ICAD, or control) were induced into apoptosis (apoptotic, lanes 2 and 4) or were mock treated (living, lanes 1 and 3). ICAD in apoptosis is cleaved by caspases and loses the FLAG tag (Western, anti-FLAG antibodies; lane 4). Agarose gel electrophoresis evidences the internucleosomal cleavage of chromosomal DNA in apoptotic wild type cells (lane 2), and its inhibition in apoptotic ICAD-expressing cells (lane 4). ICAD-expressing apoptotic cells were permeabilised, fixed and stained for DNA, HMGB1 and TUNEL. While all cells are TUNEL-negative, HMGB1 was firmly

retained into the nucleus of the cell showing chromatin condensation (in upper right corner). d, Total extracts from about 5 million living and apoptotic HeLa cells were subjected to 2-D electrophoresis and immunoblotted with anti-HMGB1 antibodies. Multiple acetylated forms of HMGB1 are visible, but no difference is detectable between the two samples. e, Histone H4 in apoptotic cells is hypoacetylated. Immunoblotting was performed with R10 antibody (specific for the acetylated forms of H4).

Figure 4 HMGB1 release promotes inflammatory responses. a, Necrotic cells lacking HMGB1 do not elicit the production of the proinflammatory TNF-α cytokine by monocytes. Bars represent standard errors (n=3). b, Apoptotic cells undergoing secondary necrosis and partial autolysis do not promote inflammatory responses, unless HMGB1 is mobilized by treatment with TSA. The experiment was repeated 3 times in duplicate, with 2 different amounts of apoptotic cells to ensure linearity in TNF-α production. Values are normalized to a value of 1 for the amount of TNF-α added after challenge with 0.2×10^5 apoptotic cells. c, Anti-HMGB1 antibodies reduce inflammation in liver injured by acetaminophen (AAP) overdose. Liver injury (alanine transaminase activity in serum) and inflammatory cell recruitment (myeloperoxidase activity in total liver extracts) was assed after 9 hours. MPO/ALT ratios indicate inflammation normalized to liver damage. Each point represents one mouse, the bar indicates the median value, and the grey shade indicates the area included within average $\pm$ standard error. Pairwise comparisons (Mann-Whitney test) between the groups of mice are indicated by the arrows.

Figure 5 HMGB1 chemotactic assay on staminal (stem) cells. Chemotaxis assays were performed using modified Boyden chambers. The value of 1corresponds to the number of cells migrating in the absence of any stimulator (random cell migration).
The data represent the mean ±SE. The statistical significance of the result is $P<0.0001$ in an ANOVA model. Treatment with HMGB1 plus anti–HMGB1 gave results that did not differ statistically from the unstimulated control. Treatment of D18 cells with the anti–HMG1 antibody alone, or an unspecific antibody, also were indistinguishable from the unstimulated control.

Figure 6 Growth curve of staminal (stem) cells in the presence of HMGB1. 5x104 D18 cells were plated in 3 cm wells and grown for 24 hours at 37°C in 5% CO_2 in RPMI supplemented with 20% fetal bovine serum (FBS). Medium was then replaced with RPMI (no serum) for 16 hours. Subsequently the medium was replaced with fresh media containing RPMI alone, RPMI plus 20% FBS, and RPMI with HMGB1 at the indicated concentrations (no serum). Cells were harvested at the indicated times (days 1, 2 and 3) and counted with hemacytometer. Cells in RPMI alone did not divide, while cells in RPMI plus HMGB1 divided actively for at least 24 hours, and then more slowly due to nutrient exhaustion. At day 3, nonetheless, all plates with D18 cells stimulated with HMGB1 (all concentrations) contained some dividing cells, as evaluated by microscopic inspection.

HMGB1 is loosely bound to chromatin of both interphase and mitotic cells, and it is rapidly leaked out into the medium when membrane integrity is lost in permeabilized or necrotic cells[7-9]. These results suggested that in living cells HMGB1 associates and dissociates rapidly from chromatin. To prove this, we tagged HMGB1 with GFP at its C terminus, forming a chimeric protein that was equivalent to the unperturbed HMGB1 in enhancing the expression of a HOXD9-responsive reporter gene in transfection assays[10] (results not shown). HeLa cells expressing the fusion protein were easily detectable by the uniform green fluorescence of their nuclei. Cells undergoing mitosis showed a diffuse cytoplasmic fluorescence, but also a distinct association of HMGB1-GFP to condensed chromosomes, that lasted throughout M phase (Fig. 1a and data not shown).

When HMGB1-GFP transfected HeLa cells were permeabilized with NP-40, most lost their fluorescence after a few seconds, confirming the loose association of HMGB1 to chromatin. However, a few cells retained a bright fluorescence. From the characteristically fragmented appearance of their nuclei, these cells appeared apoptotic. We then forced HeLa cells to undergo apoptosis by treatment with TNF-α and cycloheximide, we permeabilized them, and we immunostained them for their

endogenous, unmodified HMGB1. Whereas control non-apoptotic cells leaked all HMGB1 into the medium (Fig. 1b,d), the protein was retained within the nucleus of apoptotic cells (Fig. 1d). HMGB1 was mostly retained associated with nuclear remnants even after prolonged incubation and partial autolysis of apoptotic cells, when soluble cytoplasmic proteins like lactate dehydrogenase (LDH) leaked into the extracellular medium (Fig. 1e). HMGB1 and HMGB1-GFP also bound tightly to chromatin in HeLa and 3T3 cells induced into apoptosis by etoposide or H_2O_2 treatment, or apoptosing spontaneously in unperturbed cultures (data not shown). In contrast, HMGB1 dissociated from the chromatin of necrotic cells and leaked to the extracellular medium (Fig. 1c).

To quantify the dynamic properties of HMGB1-GFP within single cells, we used a technique called fluorescence loss in photobleaching (FLIP)[11]. Repeated bleaching of the same area leads to fluorescence loss from the rest of the nucleus, with kinetics dependent on the overall mobility of the fluorescent protein. If a fraction of the protein pool is at any given time bound to chromatin, the loss of its fluorescence will be slowed. Bleaching of total nuclear HMGB1-GFP was rapidly obtained (Fig. 2a); in contrast, in HeLa cells expressing GFP fusions to chromatin proteins HMGN1 and HMGN2, or transcription factor NF1, fluorescence loss was significantly slower; in cells expressing GFP-histone H1c fusions, fluorescence loss was very limited (Fig. 2c and ref. 11).

We also assessed the diffusion rate of HMGB1-GFP associated to condensed chromosomes of living HeLa cells during mitosis. Repeated bleaching of cytoplasmic HMGB1-GFP led to rapid and parallel loss of fluorescence from condensed chromosomes and from the cytoplasm (Fig. 2b,d), proving unequivocally that HMGB1 turns over fast between the chromatin-bound and soluble states.

To the other extreme, HMGB1-GFP appeared almost immobile in apoptotic cells (Fig. 2e,g). The blockade of HMGB1 is specific, since the mobility of GFP-HMGN1, GFP-HMGN2, GFP-NF1 and GFP alone is not reduced in apoptotic cells as opposed to living ones (Fig. 2h and results not shown). Thus, chromatin condensation during apoptosis does not impair protein mobility in general.

Hmgb1$^{-/-}$ cells offered us the opportunity to test whether the binding of HMGB1 to apoptotic chromatin was due to alterations of HMGB1, or of nuclei undergoing apoptosis. Embryonic fibroblasts obtained from *Hmgb1*$^{-/-}$ and *Hmgb1*$^{+/+}$ mice[13] were equally susceptible to apoptosis (not shown), indicating that freezing of HMGB1 onto chromatin is a consequence of apoptosis, but not a requisite. *Hmgb1*$^{-/-}$ fibroblasts were treated with TNF- and cycloheximide, and apoptotic cells were recovered from the flask by gentle flushing. This cell population, and a control population of non-apoptotic *Hmgb1*$^{-/-}$ fibroblasts, were permeabilized with detergent and exposed to bacterially produced, Cy5-labelled HMGB1. HMGB1 bound to apoptotic nuclei, but not to non-apoptotic ones (Fig. 3a). This result was confirmed biochemically (Fig. 3b): permeabilized apoptotic and non-apoptotic *Hmgb1*$^{-/-}$ fibroblasts were incubated with bacterially made HMGB1 and fractionated through a discontinuous sucrose gradient[14]. Again, HMGB1 associated to the nuclei from apoptotic cells, but not to the ones from non-apoptotic cells. The experiments described above indicate that, upon apoptosis, chromatin undergoes some chemical or structural transition that makes it susceptible to HMGB1 binding. The nature of HMGB1 itself, whether endogenous or made in bacteria, tagged with fluorophores or fused to GFP, is irrelevant.

We next investigated the nature of the chromatin modification allowing the stable binding of HMGB1. Since HMGB1 binds tightly to in vitro reconstructed mononucleosomes[9,15], we tested whether the fragmentation of chromatin to oligo- and mononucleosomes that occurs in the later stages of apoptosis would provide stable binding sites for HMGB1. HeLa cells were stably transfected with a construct expressing ICAD, the inhibitor of the CAD nuclease that fragments DNA during apoptosis. HeLa cells overexpressing ICAD underwent apoptosis, but their DNA showed little if any fragmentation[16] (Fig. 3c). HMGB1 bound equally stably to ICAD-expressing, nonfragmented chromatin, and to fragmented chromatin (Fig. 3c, and results not shown). DNA fragmentation therefore cannot account for stable HMGB1 binding in apoptosis.

Alteration of the acetylation status of chromatin was tested next. TSA, a general deacetylase inhibitor, was added to the medium of HeLa cells just prior to the induction of apoptosis: in this case, HMGB1 freezing onto chromatin was suppressed (Fig. 2f,g). This result suggests that hypoacetylation of one or more chromatin components occurs during apoptosis, and favors HMGB1 binding. No difference was seen in the pI or the molecular weight pattern of HMGB1 present in apoptotic and non-apoptotic cells (Fig. 3d), indicating that apoptosis was not changing the acetylation status of HMGB1 itself. In contrast, histone H4 from apoptotic chromatin is notably hypoacetylated in comparison to non-apoptotic chromatin, and H4 hypoacetylation in apoptosis is suppressed by TSA (Fig. 3e).

We thus showed that HMGB1 binding to chromatin depends on the viability of the cell, and clearly distinguishes necrotic from apoptotic cells. We then reasoned that the differential release of HMGB1 might be exploited as a cue to nearby cells to activate the appropriate responses to unprogrammed and programmed cell death. Unprogrammed death is usually the result of trauma, poisoning or infection, all events that require prompt reaction and damage containment and/or repair. Inflammation is the primary tissue damage response in mammals, and HMGB1 has already been reported to be a mediator of inflammation[4-6]. To test directly whether the release of HMGB1 by necrotic cells can be the immediate trigger for an inflammatory response, we challenged wild type bone marrow cells with *Hmgb1*$^{-/-}$ or wild type (+/+) dead fibroblasts. As expected[18], wild type necrotic cells triggered the production of the proinflammatory cytokine TNF-α, whereas wild type apoptotic cells were much less effective (Fig. 4a). Significantly, *Hmgb1*$^{-/-}$ necrotic cells were also rather ineffective in activating monocytes. Purified HMGB1 also elicits TNF-α production in this assay (ref. 6 and results not shown). Thus, this experiment shows that HMGB1 is one of the major diffusible signals of necrosis. On the other hand, it cannot test whether apoptotic cells escape the inflammatory surveillance because they retain HMGB1: apoptotic cells start to leak out cellular components only after several hours, and in vivo they are routinely cleared by phagocytic cells well before this process (termed secondary necrosis) can take place. We could nonetheless test whether cells undergoing post-apoptotic,

secondary necrosis are able to promote inflammatory responses in monocytes. Wild type, apoptotic fibroblasts were incubated with for 72 hours, until most LDH was released in the extracellular medium; the post-apoptotic cell remnants did not promote a strong inflammatory response in monocytes (Fig. 4b). However, fibroblasts treated with TSA while undergoing apoptosis generated secondarily necrotic cell remnants that promoted inflammation as vigorously as primary necrotic cells killed by freeze-thawing.

We could not test whether *Hmgb1*$^{-/-}$ mice have a reduced inflammatory response following tissue necrosis, because these mice survive only a few hours after birth[13]. To provide evidence in an animal model for the significance of HMGB1 release, we induced massive hepatocyte necrosis in wild type mice and measured the inflammatory response. Both in humans and rodents, an overdose of the analgesic acetaminophen (AAP, also known as paracetamol) produces large areas of liver necrosis, concomitant with local inflammation, Kupffer cell activation and the recruitment and sequestration of neutrophils and macrophages into the injured tissue[19,20]. Levels of liver damage and neutrophil sequestration are strictly proportional until most hepatocytes become necrotic between 12 and 24 hours after AAP poisoning[20]. We administered 300 mg/kg AAP with a single intraperitoneal injection to young mice, and 9 hours later estimated liver injury by measuring alanine transaminase (ALT) activity in serum, and inflammatory cell sequestration by measuring myeloperoxidase (MPO) activity in total liver extracts. One group of mice (n=8) received no AAP, one group (n=10) received AAP alone, another (n=6) AAP and affinity purified anti-HMGB1 antibodies (300 mg/kg), and the last one (n=8) AAP and irrelevant rabbit antibodies (300 mg/kg). All 3 groups injected with AAP had elevated ALT levels in comparison to sham-treated controls, but the differences between the 3 treated groups were not statistically significant. Thus, antibodies do not protect against liver damage, at least at the onset of the inflammatory response. We then used the MPO/ALT ratio to compare inflammatory cell recruitment, normalized to the level of liver damage (Fig. 4c). Anti-HMGB1 antibodies were effective in reducing inflammation following AAP-induced liver necrosis: these mice showed a significantly reduced MPO/ALT ratio (1.5 ± 0.3) both in comparison to mice

injected with AAP alone (2.7± 0.3; p<0.05), and to mice injected with AAP and preimmune rabbit IgGs (2.4± 0.3; p<0.05). No HMGB1 can derive from activated monocytes and macrophages in our experiment, because HMGB1 secretion from inflammatory cells requires at least 16 hours[4,6]. Thus, HMGB1 acts as an immediate trigger of inflammation, as well as a late mediator of inflammation as previously described[4].

In conclusion, we have shown that the passive release of an abundant chromatin component can serve as a diffusible signal of *unprogrammed* death, that can be used as a cue to nearby cells. Core histones, though more abundant, would probably not be good signals of necrosis, as they remain anchored to the insoluble chromatin of necrotic cells. Apoptotic cells are not the result of a present and immediate danger and do not trigger inflammation in physiological conditions. They retain nuclear components until cleared by macrophages or nearby cells that act as semi-professional phagocytes, that they attract and activate by displaying "eat me" signals[21]. However, apoptotic cells that escape prompt clearance undergo secondary necrosis, lead to an increased level of nuclear autoantibodies[22], and have been proposed to play an important pathogenetic role in autoimmune diseases, such as lupus[23]. Thus, the retention of HMGB1 by apoptotic cells undergoing secondary necrosis represents an additional safeguard against confusing necrotic and apoptotic cells.

The author also demonstrated that HMGB1 has chemotactic activity on D18 mouse mesoangioblasts derived from fetal aorta cells (Minasi et al. Development. 2002 Jun;129(11):2773-83.). Mesoangioblastic stem cells can be derived from mouse fetal aorta cells but also from umbilical chord cells, peripheral blood vessels and bone marrow ckit+ cells in post-natal mice. Once derived from these original cell populations with a proprietary method (described in Minasi et al.) mesangioblastic cells, of which D18 are an example, are 'naturally'immortalized (they grow indefinitely). D18 cells can be indefinitely expanded in vitro and remain pluripotent in vitro and in vivo, serving as precursors of the following mesodermal tissue types: bone, cartilage, skeletal smooth and cardiac muscle, endothelial cells, monocytes, macrophages and osteoclasts. They

are also capable of generating hepatocytes and neurons. Clone D18 was deposited according to the Budapest Treaty at CBA, Centro Biotecnologie Avanzate, Genova, Italy, N. PDO2005). Chemotaxis assays were performed using modified Boyden chambers. The value of 1 corresponds to the number of cells migrating in the absence of any stimulator (random cell migration).

The data represent the mean ±SE. The statistical significance of the result is $P<0.0001$ in an ANOVA model. Treatment with HMGB1 plus anti–HMGB1 gave results that did not differ statistically from the unstimulated control. Treatment of D18 cells with the anti–HMG1 antibody alone, or an unspecific antibody, also were indistinguishable from the unstimulated control.

Stem cells are expected to proliferate at the site where tissue repair must take place. We then tested whether HMGB1 could also stimutate stem cell proliferation. Stem cells in RPMI with no serum did not divide, while cells in RPMI with no serum but in the presence of HMGB1 divided actively for at least 24 hours, and then more slowly due to nutrient exhaustion. At day 3, nonetheless, all plates with D18 cells stimulated with HMGB1 (at all concentrations) contained some dividing cells, as evaluated by microscopic inspection.

Methods

Nomenclature. High mobility group proteins have been renamed recently[24]. High mobility group 1 protein is now officially designated as HMGB1; alternative names are HMG1, amphoterin, and p30. HMG-14 and HMG-17 are now HMGN1 and HMGN2.

Cloning, expression and purification of HMGB1 protein and fragments. The plasmids pRNHMG1/M1-V176 to express boxA+boxB, pT7-HMG1bA encoding box A and pT7-HMG1bB encoding box B have been described (Bianchi, M. E., Falciola, L., Ferrari, S., and Lilley, D. M. J. (1992) EMBO J. 11, 1055-1063). The plasmid pT7-7-rHMG1cm encoding the full-length HMGB1 protein was a kind gift of Prof. J.O. Thomas (Cambridge). The construct pT7-HMGB1 AB_{bt}, encoding amino

acids 1-187, was created by replacing the NsiI-HindIII fragment of the plasmid pT7-RNHMG1 (Bianchi, M. E., Falciola, L., Ferrari, S., and Lilley, D. M. J. (1992) EMBO J. 11, 1055-1063) with a PCR product between the primer pair 5'-CATATGCATTCTTTGTGCAAACCT-3' and 5'-CCCCAAGCTTATTCCTTCTTTTTCTTG CTC-3', cut with *Nsi*I and *Hin*dIII. The structure of the construct was verified by nucleotide sequencing. Expression and purification of the single boxes and HMGB1 (AB) was performed as described previously and HMGB1 AB_{bt} was purified accordingly (Bianchi, M. E., Falciola, L., Ferrari, S., and Lilley, D. M. J. (1992) EMBO J. 11, 1055-1063). For the full-length protein, a freshly transformed colony was used to inoculate an overnight culture grown in M9 medium complemented with 20 g/l cas-aminoacids, 0.5% glycerol, 5 g/l yeast extract, 0.4% glucose and 100 µg/ml chloramphenicol. 300 ml of the overnight culture were than used to inoculate a 3 l culture in an ADI 7 l autoclavable bioreactor (Applikon). At an optical density OD595 of 0.7, IPTG was added to the culture to a final concentration of 0.5 mM. Shaking was reduced to 150-200 rpm and growth temperature to 23°C. Growth was continued for another 16 hours. The protein was processed as described above, and we obtained 25 mg pure full-length HMGB1. Protein concentrations were determined spectroscopically using the method of Gill and Hippel (Gill, S.C., and von Hippel, P.H. (1989) Analytical Biochemistry 182, 319-326). The following extinction coefficients have been used for the native protein: Box A: ε_{280}= 9.98 10^3 M^{-1} cm^{-1}, Box B: ε_{280}= 1.15 10^4 M^{-1} cm^{-1}, AB, AB_{bt} and full-length HMGB1: ε_{280}= 2.14 10^4 M^{-1} cm^{-1}.

Constructs and cells. Plasmid pEGFP-HMGB1 was generated by inserting the coding sequence of the cDNA for rat HMGB1 into pEGFP-N1 (Clontech) using the *Eco*RI and *Sac*II restriction sites. pEGFP-H1c, pEGFP-NF1, pEGFP-HMGN2, and pEF-flag-mICAD were generously provided by A. Gunjan, N. Bhattacharyya, R. Hock, M. Bustin and S. Nagata[11,12,16]. HeLa cells and fibroblasts (line VA1, *Hmgb1*$^{+/+}$, and line C1, *Hmgb1*$^{-/-}$) were grown as described[7,13]. HeLa cells were electroporated with pEGFP-HMGB1 and were observed 18 h later. The average amount of HMGB1-GFP in the cell

population was between 1 and 3% of HMGB1 (by immunoblotting with anti-HMGB1 antibodies). At the single cell level, the amount of HMGB1-GFP varied at most tenfold between different cells; care was taken to always use for analysis cells with a moderate fluorescence level.

Apoptosis was induced by treating the cells for 16 h with 2 ng/ml human TNF-α and 35 μM cycloheximide. Necrosis was induced by treatment for 16 h either with 5 μM ionomycin and 20 μM CCCP, or 6 mM deoxyglucose and 10 mM sodium azide, or by 3 cycles of freezing and thawing.

Three different clones of HeLa cells stably transfected with pEF-flag-mICAD were stained with TUNEL (Apoptosis detection system, Promega), and their chromosomal DNA was extracted and electrophoresed on a 1.5% agarose gel.

Indirect immunofluorescence was performed as described[7] using an anti-HMGB1 polyclonal antibody (Pharmingen) at 1:1600 dilution, and FITC- or TRIC-conjugated anti-rabbit antibodies (Boehringer) at 1:300 dilution.

In vivo microscopy and FLIP. Cells were plated and observed in LabTek II chambers (Nalgene) with an Axiovert 135M microscope (Zeiss). FLIP experiments were carried out on a Leica TCS-SP confocal microscope using the 488 nm excitation line of an Ar laser and detection at 500-575 nm as described[11]. Cells were bleached (in a spot of 1 μm in radius, 20 mW nominal output, 200-500 ms) and imaged (at 0.2 mW nominal output) at intervals of 6 s.

Binding of recombinant HMGB1 to chromatin. *Hmgb1*$^{-/-}$ fibroblasts were treated with 2 ng/ml hTNF-α and 35 μM cycloheximide. After 16 h, apoptotic cells were recovered by gentle flushing of the dish. Ten million apoptotic *Hmgb1*$^{-/-}$ fibroblasts and a control population of non-apoptotic ones were resuspended in 50 μl PBS containing 0.32 M sucrose, 0.5% NP-40 and 1 μM bacterially produced HMGB1, either fluorescently labelled with Cy5 (Pharmacia) or unlabelled. Average labelling was 2.3 Cy5 molecules per HMGB1 molecule. After 30 minutes at room temperature, sample cells were mixed and mounted on slides using Vectashield (Vector Laboratories) containing 1.5 μg/ml DAPI, and observed on an Axiophot microscope (Carl Zeiss) with

TRITC filter. The two pools of cells incubated with unlabelled HMGB1 were layered onto discontinuous gradients formed by 5 ml of 1.16 M sucrose in PBS and a 6 ml cushion of 2 M sucrose in PBS[14], and centrifuged at 30,000 g for 90 minutes in a SW27 Beckman rotor. Apoptotic and non-apoptotic chromatin free from membrane debris was recovered from the bottom of the tubes, and applied to a 12% SDS-PA gel. The amount of recombinant HMGB1 bound to apoptotic and non-apoptotic chromatin was determined by immunoblotting using an anti-HMGB1 antibody (Pharmingen) at 1:3000 dilution. Aliquots of apoptotic and non-apoptotic chromatin were also probed with anti-acetyl-histone H4 (R10, a gift from B. Turner), anti-acetyl-Histone H3 (Lys 9, Biolabs) and anti-acetyl-lysine antibody (Biolabs).

Inflammation assays. To measure TNF-α production in vitro, bone marrow was recovered from the hind legs of female C56Bl6 mice, diluted to $5 \cdot 10^6$ cells/ml in Optimem and dispensed in 96-well microtiter plates (120 µl per well). Necrotic cells (lysed by 3 cycles of freeze-thawing) or apoptotic cells were added to the indicated final concentration into the wells and incubated at 37°C for 18 hours. TNF-α in the supernatant was assayed by ELISA (Quantikine M, R&D Systems). TSA was added at 200 ng/ml together with TNF-α, when indicated, and was washed away before mixing the apoptotic cells with bone marrow cells.

To measure inflammation in vivo, one day old mice (weighing 1.1 ± 0.1 grams) were injected intraperitoneally with 20 µl of PBS containing 320 µg of acetaminophen (Sigma), and 320 µg of antibodies (Pharmingen BD) where indicated. After 9 hours the mice were analyzed for serum ALT activity with the GP-Transaminase kit (Sigma) and for MPO activity in liver extracts as described[25]. Statistical analysis was performed with the non-parametric Mann-Whitney test on MPO/ALT ratios. Similar results were obtained using the t-test on the MPO levels of mice paired so as to minimize the difference in ALT levels.

Chemotaxis Assay. Boyden chambers were used with filters (8-µm pore size; Corning) treated with collagen I (100 µg/ml in 0.5 M acetic acid) and fibronectin (10 µg/ml). 50,000 cells in serum-free RPMI was added to the upper well of Boyden chambers. The molecules to be tested were diluted in serum-free medium and added to the lower well.

Antibodies, pertussis toxin (PT), or inhibitor were added in both wells of Boyden chambers. Migration was allowed for 12 hours at 37°C. Then, cells remaining on the upper surface of filters were scraped off and filters were fixed in methanol and stained in a solution of 10% (wt/vol) crystal violet in 20% (vol/vol) methanol. All experiments were performed at least twice in triplicate. Results are the mean ± SE of the number of cells counted in 10 fields (40x objective) per filter and expressed as fold over control. Random cell migration (i.e., migration in the absence of chemoattractant) was given the arbitrary value of 100%.

Proliferation Assay

$5x10^4$ D18 cells were plated in 3 cm wells and grown for 24 hours at 37°C in 5% CO_2 in RPMI supplemented with 20% fetal bovine serum (FBS). Medium was then replaced with RPMI (no serum) for 16 hours. Subsequently the medium was replaced with fresh media containing RPMI alone, RPMI plus 20% FBS, and RPMI with HMGB1 at the indicated concentrations (no serum). Cells were harvested at the indicated times (days 1, 2 and 3) and counted with hemacytometer.

Cells in RPMI alone did not divide, while cells in RPMI plus HMGB1 divided actively for at least 24 hours, and then more slowly due to nutrient exhaustion. At day 3, nonetheless, all plates with D18 cells stimulated with HMGB1 (all concentrations) contained some dividing cells, as evaluated by microscopic inspection (Fig. 6).

The experiment has been repeated with mesangioblasts derived from adult mouse capillaries (stem cells from adult), showing the same proliferation inducing effect.

According to the above experimental data, the present invention refers to a method to induce stem cell migration and/or proliferation in cell culture or *in vivo* comprising the step of exposing such cells to effective amounts of the HMGB1 protein or functional parts thereof. For the instant invention the term "stem cells" refers to either staminal, pluripotent precursor or progenitor cells.

Moreover the invention refers to compositions comprising the HMGB1 protein or functional parts thereof to be used for the treatment of first step of inflammation processes, preferably together with a different anti-inflammation agent.

Moreover the invention refers to antagonists of HMGB1 protein or functional parts thereof able to act at the first stage of the inflammation process and in case of large scale necrosis such as intestinal infarction, acute pancreatitis and extensive trauma. Antagonists comprise but are not limited to HMGB1 antibodies or functional recombinant or synthetic portions thereof.

Moreover the invention refers to compositions comprising the HMGB1 protein or functional parts thereof to be used for the treatment of tissue repair and/or regeneration, preferably together with an anti-inflammation agent. Conveniently the composition may be perfused at the tissue repair site.

A tissue repair and/or regeneration site comprises but is not limited to trauma sites, ischemia sites, burn sites.

Bibliography

1. Bustin, M. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Mol. Cell. Biol.* **19**, 5237-5246 (1999).
2. Bianchi, M. E. & Beltrame, M. Upwardly mobile proteins. The role of HMG proteins in chromatin structure, gene expression and neoplasia. *EMBO Reports* **1**, 109-114 (2000).
3. Hori, O. et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. J. Biol. Chem. 270, 25752-25761 (1995).
4. Wang, H. et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 285, 248-251 (1999).
5. Abraham, E., Arcaroli, J., Carmody, A., Wang, H. & Tracey, K. J. HMG-1 as a mediator of acute lung inflammation. J. Immunol. 165, 2950-2954 (2000).
6. Andersson, U. et al. High Mobility Group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J. Exp. Med. 192, 565-570 (2000).

7. Degryse, B. et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J. Cell Biol. 152: 1197-2006 (2001).

8. Müller, S. et al. The double life of HMGB1 chromatin protein: architectural factor and extracellular signal. EMBO J. 16: 4337-4340 (2001).

9. Falciola, L. et al. High mobility group 1 (HMG1) protein is not stably associated with the chromosomes of somatic cells. J. Cell Biol. 137, 19-26 (1997).

10. Zappavigna, V., Falciola, L., Helmer Citterich, M., Mavilio, F. & Bianchi, M. E. HMG1 cooperates with HOX proteins in DNA binding and transcriptional activation. EMBO J. 15, 4981-4991 (1996).

11. Phair, R. D. & Misteli, T. High mobility of proteins in the mammalian cell nucleus. Nature 404, 604-609 (2000).

12. Misteli, T., Gunjan, A., Hock, R., Bustin, M. & Brown, D. T. Dynamic binding of histone H1 to chromatin in living cells. Nature 408, 877-881 (2000).

13. Calogero, S. et al. The lack of chromosomal protein HMG1 does not disrupt cell growth, but causes lethal hypoglycaemia in newborn mice. Nature Genet. 22, 276-280 (1999).

14. Bender, T. P. in Current protocols in molecular biology (eds. Ausubel, F. et al.) Current Protocols (1994).

15. Nightingale, K., Dimitrov, S., Reeves, R. & Wolffe, A. P. Evidence for a shared structural role for HMG1 and linker histones B4 and H1 in organizing chromatin. EMBO J. 15, 548-561 (1996).

16. Enari, M. et al. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43-50 (1998).

17. Misteli, T. Protein dynamics: implications for nuclear architecture and gene expression. Science 291, 843-847 (2001).

18. Fadok, V. A., Bratton, D. L., Guthrie, L. & Henson P. M. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. J. Immunol. 166, 6847-6864 (2001).

19. Thomas, S. H. L. Paracetamol (acetaminophen) poisoning. Pharmacol. Ther. 60, 91-120 (1993).

20. Lawson, J. A., Farhood, A., Hopper, R. D., Bajt, M. L. & Jaeschke, H. The hepatic inflammatory response after acetaminophen overdose: role of neutrophils. Toxicol Sci. 54, 509-516 (2000).

21. Ren, Y. & Savill, J. Apoptosis: the importance of being eaten. Cell Death Different. 5, 563-568 (1998).

22. Scott, R. S. et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature 211, 201-211 (2001).

23. Herrmann, M. et al. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Reum. 41: 1241-1250 (1998).

24. Bustin, M. Revised nomenclature for high mobility group (HMG) chromosomal proteins. Trends Biochem. Sci. 26, 152-153 (2001).

25. Kato, A., Yoshidome, H., Edwards, M. J. & Lentsch, A. B. Regulation of liver inflammatory injury by signal transducer and activator of transcription-6. Am. J. Pathol. 157: 297-302 (2000).

Docket #

DECLARATION AND POWER OF ATTORNEY

We, the below named inventors, hereby declare that:

Our residences, post office addresses, and citizenships are as stated below next to our respective names.

We believe we are the original, first, and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled, the specification of which is attached hereto.

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

We acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

We hereby declare that all statements are made hereby of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And we hereby appoint:

Maurice E. Gauthier	-	Reg. No. 20,798
Richard L. Stevens	-	Reg. No. 24,445
Matthew E. Connors	-	Reg. No. 33,298
William E. Hilton	-	Reg. No. 35,192
Patrick J. O'Shea	-	Reg. No. 35,305
Arlene J. Powers	-	Reg. No. 35,985
Richard L. Stevens, Jr.	-	Reg. No. 44,357
Peter Stecher		Reg. No. 47,259

all of the firm of Samuels, Gauthier & Stevens, our attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

We request that all correspondence be directed to:

Samuels, Gauthier & Stevens, LLP
225 Franklin Street, Suite 3300
Boston, MA 02110

Attn: Arlene J. Powers

MARCO EMILIO GIUSEPPE BIANCHI
(Full Name of Inventor)

marco bianchi
(Inventor's Signature)

16. June. 2002
(Date)

Viale ABRUZZI 4/C
20068 PESCHIERA BORROMEO
(Residence)

Italian
(Citizenship)

(Post Office Address)

(Full Name of Inventor)

(Inventor's Signature)

(Date)

(Residence)

(Citizenship)

(Post Office Address)

(Full Name of Inventor)

(Inventor's Signature)

(Date)

(Residence)

(Citizenship)

(Post Office Address)

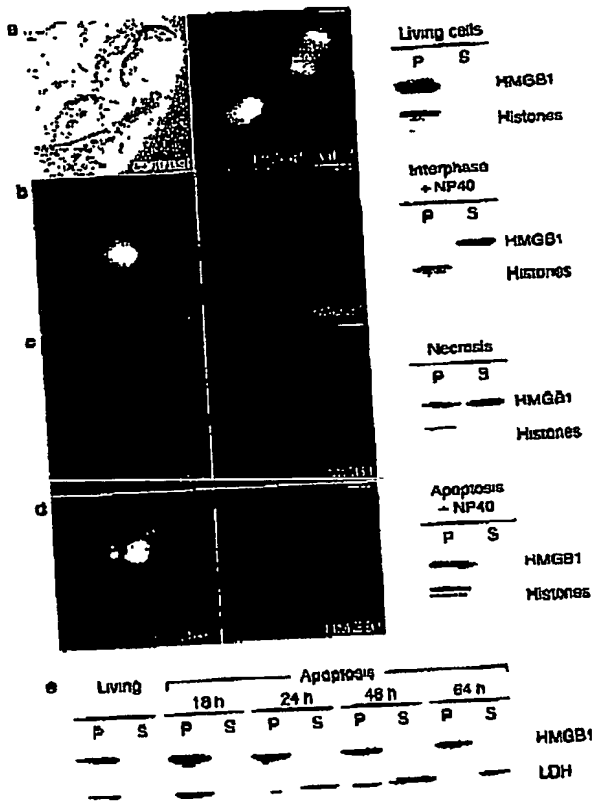

Fig.1 Scaffidi et al.

manuscript BOS619C
Adobe Photoshop 3.0.5/TIFF

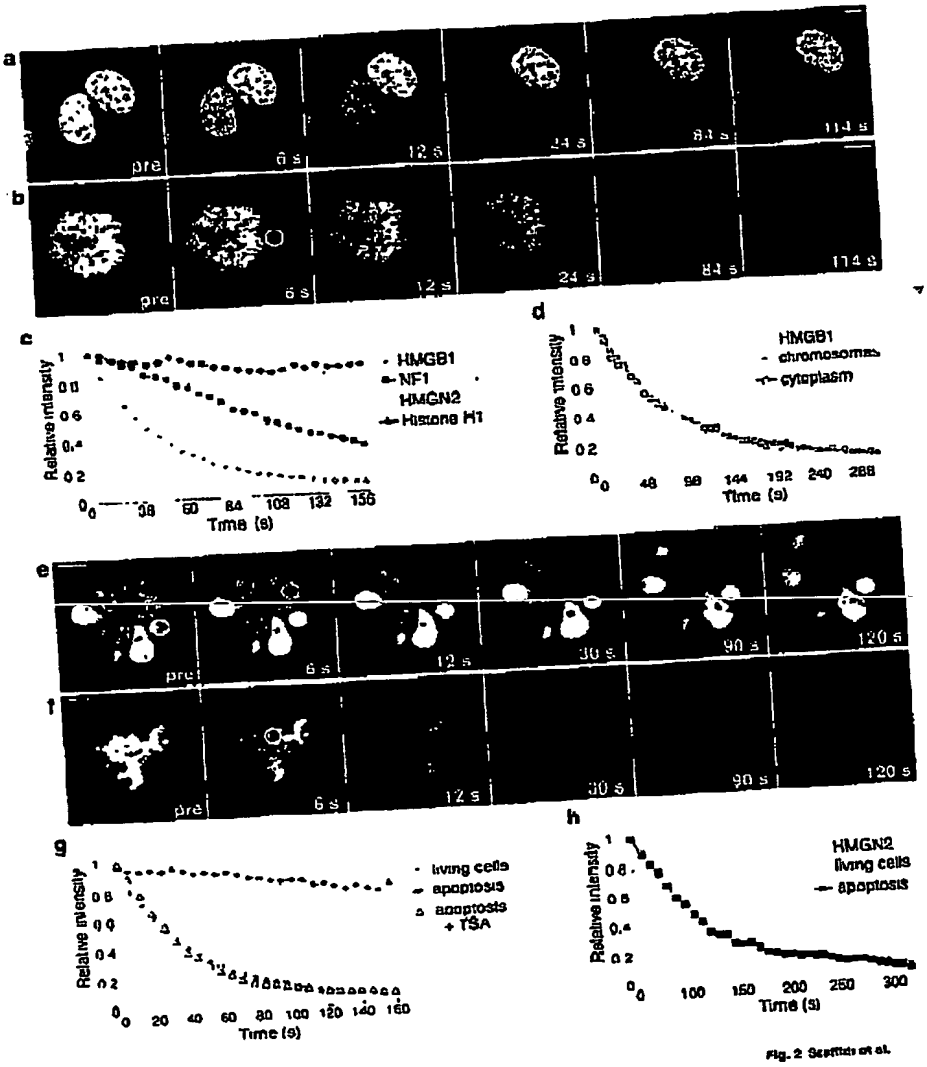

Fig. 2 Scaffidi et al.

manuscript B05619C
Adobe Photoshop 3 0.5/TIFF

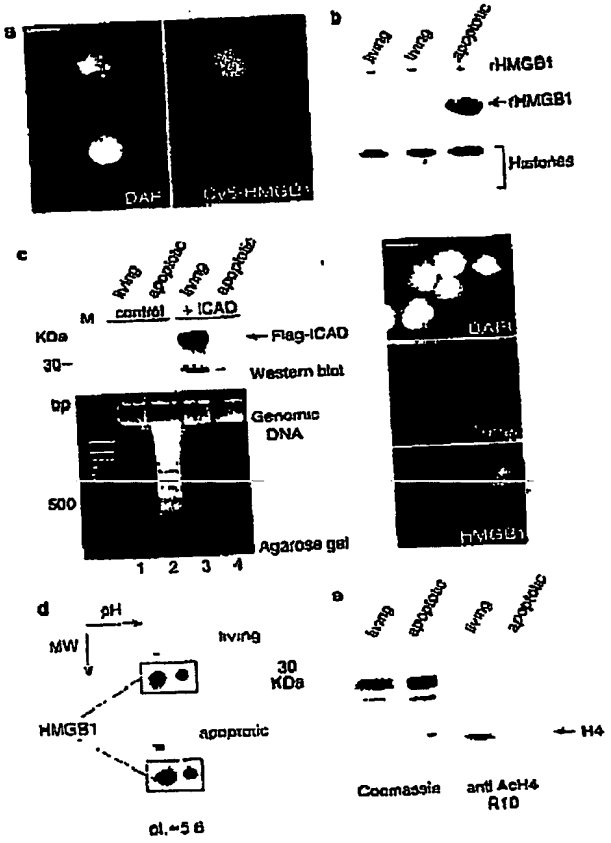

Fig. 3 Scaffidi et al.

manuscript B05619C
Adobe Photoshop 3.0.5/TIFF

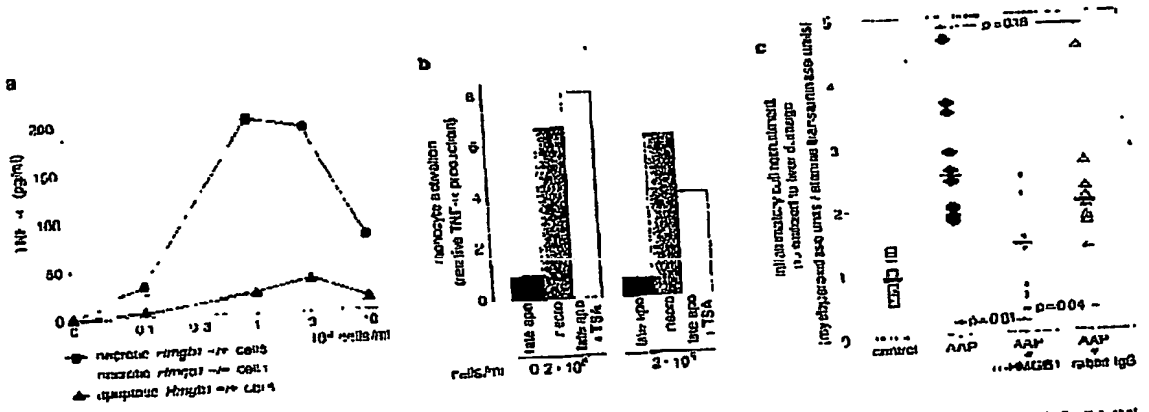

Fig. 4 Scaffold et al.
manuscript 805819C
Adobe Photoshop 3.0.5/TIFF

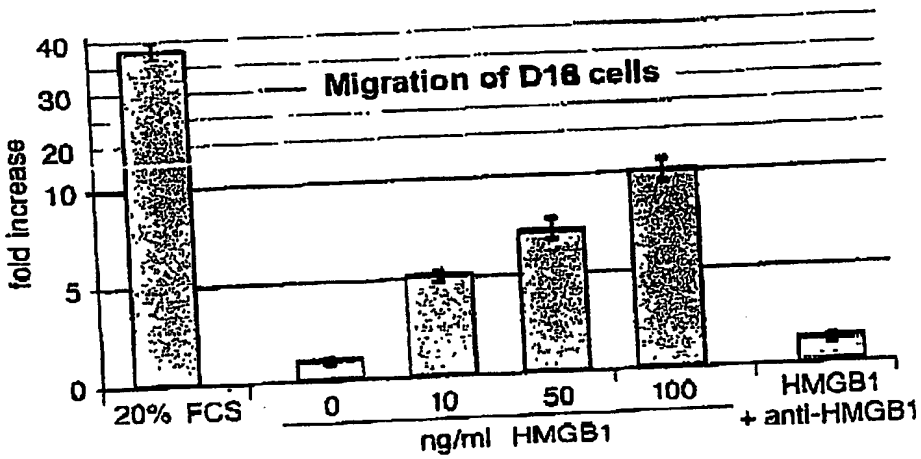
Migration of D18 cells
fold increase
40
30
20
10
5
0
20% FCS
0
10
50
100
HMGB1
+ anti-HMGB1
ng/ml HMGB1

Fig 5

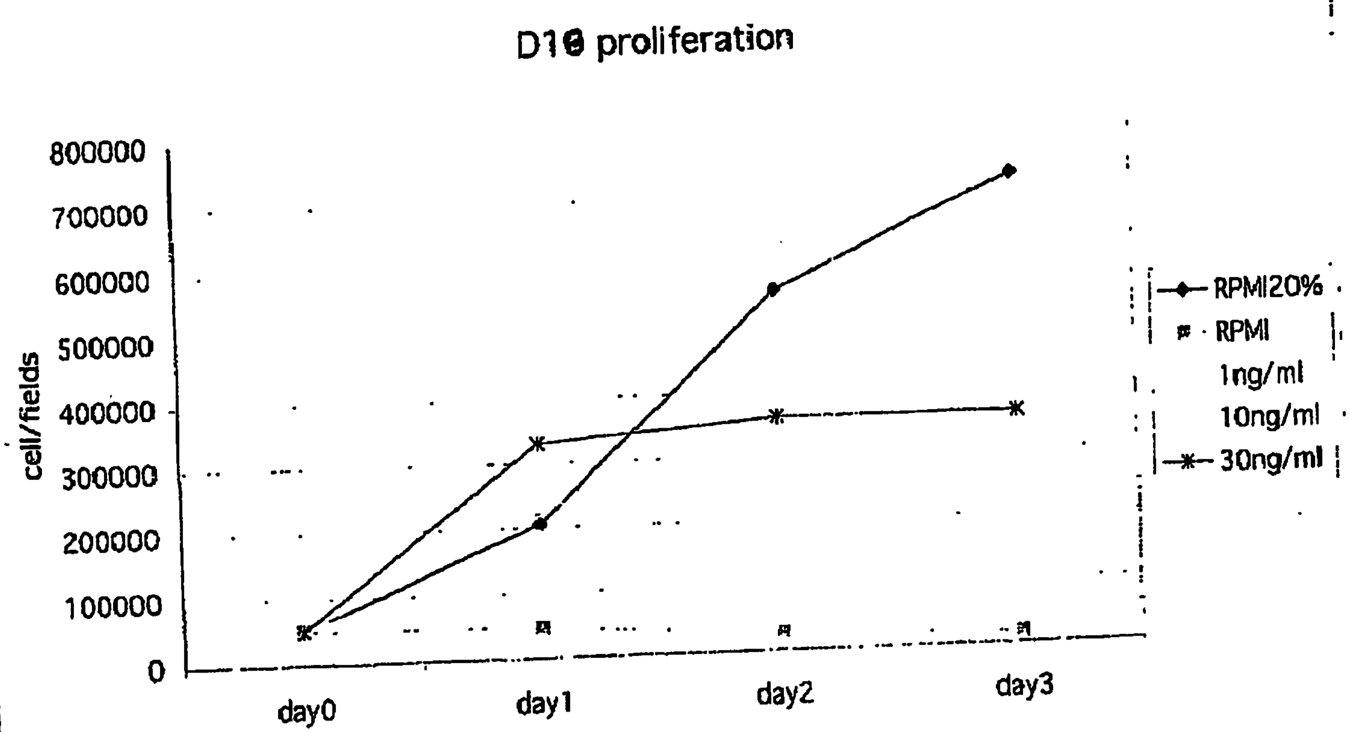
D1■ proliferation
800000
700000
600000
500000
400000
300000
200000
100000
0
cell/fields
day0
day1
day2
day3
RPMI20%
RPMI
1ng/ml
10ng/ml
30ng/ml

Fig. 6